XXXIII. THE COLORIMETRIC DETERMINATION OF PHOSPHORUS.

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Of the several colorimetric methods for the determination of phosphorus which have been described during recent years, probably the Briggs [1922] modification of the Bell and Doisy [1920] method and the method of Fiske and Subbarow [1925] have met with most general favour. Both these methods depend on the reduction of phosphomolybdic acid to give a blue colour, the intensity of which is proportional to the concentration of phosphate.

Martland and Robison [1926] proposed a very convenient modification of the Briggs procedure, in which the colour was allowed to develop at an acidity higher than that usually used. Although the total amount of colour was not as great as that developed at lower acidities, a much greater variation in acidity was allowable without any appreciable variation in the amount of colour produced. Consequently, there was no necessity of adjusting the acidity of the test solution, or of adding trichloroacetic acid to the standard to make it similar to the test in acidity and composition. By keeping the sulphuric acid and ammonium molybdate separate they were able to use the same solutions for "free" and for "total" phosphorus. In the latter case the sample was digested in a boiling-tube with the amount of sulphuric acid (1 cc. of 30 %) used in bringing up the colour, and the oxidation completed by adding a drop of 30 % hydrogen peroxide. The small amount of sulphuric acid lost during the digestion was not sufficient to cause any disproportionality in the colour produced, so that it was only necessary to wash the contents of the tube into a 15 cc. volumetric flask and add the 1 cc. of 10 % ammonium molybdate and 1 cc. of 0·5 % quinol in 20 % sodium sulphite to bring up the blue colour, which was usually read after 20 or 30 minutes.

Fiske and Subbarow pointed out several objections to the use of quinol as the reducing agent, such as the necessity of allowing 30 minutes for the colour to develop, and the lack of proportionality of colour produced with solutions of different strength in the Briggs procedure. They proposed the use of 1:2:4-aminonaphtholsulphonic acid, which has the great advantages of giving the full complement of blue colour in 5 minutes or less, and of giving excellent proportionality of colour over a wide range of concentration of phosphate ion. It has the added advantage, moreover, of being far less affected by various

substances, *e.g.* ammonium and iron salts, nitrites and nitrates, chlorides and silicates, all of which interfere in varying ways with the colour production when quinol is used as the reducing agent.

The method in use in this laboratory is a combination of the Martland and Robison and the Fiske and Subbarow procedures and is believed to have advantages over the methods previously described. The high acidity recommended by Martland and Robison has been retained, and the acid and molybdate are kept separate. The reducing agent used is the aminonaphtholsulphonic acid suggested by Fiske and Subbarow, but is made up in a slightly different way, since it has been found impossible to keep the sulphonic acid in solution in the proportions of bisulphite and sulphite recommended.

It is in the estimation of total phosphorus, however, that the chief variation in method has been introduced. Various workers have recommended the destruction of the organic matter by sulphuric and nitric acids, sulphuric acid and hydrogen peroxide, nitric acid and nitrates, by alkaline fusion mixtures and by dry incineration. For most biochemical work the sulphuric acid and hydrogen peroxide method is probably the best. Recently, however, there have come on the market concentrated preparations of perchloric acid ($HClO_4$) which are now readily obtainable in very pure form and of a strength suitable for destruction of organic material. Perchloric acid has definite advantages as an oxidising agent which are not possessed by other acids. Its 60 % solution is a heavy, colourless liquid, much of the appearance of concentrated sulphuric acid, though not so viscous as not to be easily used with a pipette. It distills at something over 200°, and is a much better oxidising agent at high temperature than is sulphuric acid, having four available oxygen atoms, while sulphuric acid has only one. At room temperatures it is practically inert, showing no tendency to oxidise materials unless strongly heated. Its formula weight is nearly the same as that of sulphuric acid, but since it is univalent, while sulphuric is bivalent, twice the amount of perchloric acid is used to give the same acidity. This extra bulk of acid has a definite advantage in the oxidation of organic matter in a boiling-tube, since there is less danger of superheating both in the liquid and at the glass surface just above the acid layer; bumping and spattering are consequently much less apt to occur than when sulphuric acid is used, and the etching of a ring near the bottom of the test-tube, which may be quite marked in tubes which have been used for a long time with sulphuric acid, is very seldom found with perchloric acid. The oxidation is not only smoother, but is much more rapid and complete and the use of any hydrogen peroxide is usually quite unnecessary.

Another considerable advantage in the use of perchloric acid, both for free and total phosphorus determinations, is that most of the salts of this acid are very soluble. The isolation of biological materials as their barium salts is much used in biochemical work, and the barium must, of course, be removed before an analysis can be made in which sulphuric acid is to be used. Barium perchlorate, however, is very soluble in water and by substituting perchloric for

sulphuric acid in the estimation of both the free and total phosphorus of barium salts, the necessity for removing the barium is eliminated.

Method.

Solutions required. 72 % or 60 % perchloric acid (the strength commonly obtainable on the market to-day). 1 cc. of 72 % or 1·2 cc. of 60 % perchloric acid contain almost the same "total acidity" as 1 cc. of 30 % (by volume) sulphuric acid (the amount used by Martland and Robison).

5 % ammonium molybdate.

0·2 % aminonaphtholsulphonic acid. 0·5 g. of the 1:2:4-acid, 30 g. sodium bisulphite and 6 g. crystalline sodium sulphite are dissolved by shaking with enough water to make 250 cc. If the solution does not filter clear it should be left overnight and again filtered. A fresh solution should be prepared every 2 weeks.

Standard phosphate. A stock solution is made by dissolving 2·1935 g. of pure potassium dihydrogen phosphate in 500 cc. of water. This solution contains 1·0 mg. P per cc. A dilute standard solution is made by diluting 5 cc. of the stock solution to 500 cc. with water. 10 cc. of this solution contain 0·1 mg. P. Both solutions should be kept saturated with chloroform to prevent any bacterial growth, which might otherwise cause a loss of inorganic phosphate.

Procedure.

Inorganic phosphate. An amount of the solution to be tested is measured into a 15 cc. volumetric flask and water added to about 10 cc. 1 cc. of 72 % or 1·2 cc. of 60 % perchloric acid, 1 cc. of molybdate and 0·5 cc. of sulphonic acid are added, and water to 15 cc. A standard containing an appropriate amount of phosphate (5 or 10 cc. of the dilute standard solution equivalent to 0·05 or 0·10 mg. P) is prepared at the same time and in the same way. The contents of the flasks are gently shaken between each addition, and finally mixed by inverting and shaking. The colours are read after 5 minutes in a Duboscq colorimeter.

For the determination of inorganic phosphate in trichloroacetic acid filtrates of blood[1], urine and other solutions where there is no barium present, it may be preferable to use sulphuric acid instead of perchloric acid. In this case it is convenient to use a mixed solution of 5 % ammonium molybdate in 15 % (by volume) sulphuric acid. 5 or 10 cc. of protein-free blood-filtrate, 0·2 or 0·5 cc. of urine are treated with 2 cc. of the molybdate-sulphuric acid mixture[2], 0·5 cc. of sulphonic acid and water to 15 cc.

[1] Usually prepared by shaking 1 volume of oxalated blood with 4 volumes of 10 % trichloroacetic acid and filtering. 5 cc. of filtrate represent 1 cc. of blood.

[2] It has been found advisable to employ this larger amount of molybdate, in the presence of sulphuric acid, since the colour is much slower to develop with sulphuric acid than with perchloric acid when only 1 cc. of 5 % ammonium molybdate is used. With 2 cc. of 5 % molybdate, however, the colour develops at almost the same rate in the presence of either sulphuric or perchloric acid. The smaller concentration of molybdate is used with perchloric acid because the permissible variation in acidity is greater, while the total amount of colour produced is almost the same.

Table I. *Colorimetric readings by perchloric acid method in presence of interfering substances.*

Standard 0·1 mg. P, 1·2 cc. 60 % $HClO_4$, 1 cc. 5 % ammonium molybdate, and 0·5 cc. sulphonic acid in a volume of 15 cc. Standard set at 20 mm.

Time (min.)	$HClO_4$ −0·4 cc.	$HClO_4$ −0·2 cc.	$HClO_4$ Standard amount	$HClO_4$ +0·2 cc.	Substance added: 25 % $CCl_3.CO_2H$ 1 cc.	25 % $CCl_3.CO_2H$ 2 cc.	25 % $CCl_3.CO_2H$ 3 cc.	*M* NaCl	*M*/3 $NaNO_3$	*M*/3 $(NH_4)_2SO_4$	Na_2SiO_3 5 mg. SiO_2
					0·04 mg. P (theoretical reading 50 mm.)						
2	—	50·1	50·0	—	50·0	49·9	—	—	—	—	47·0
5	47·5	50·1	49·2	50·2	50·1	50·1	—	—	—	—	46·6
10	47·1	50·9	49·2	—	50·1	50·0	—	—	—	—	46·6
15	47·3	51·3	—	50·4	49·9	49·8	—	—	—	—	—
20	47·2	51·1	49·2	50·3	50·1	49·8	—	—	—	—	—
30	46·5	50·6	—	50·2	50·1	49·9	—	—	—	—	—
					0·08 mg. P (theoretical reading 25 mm.) +50 mg. per 100 cc. $K_2C_2O_4$						
2	25·6	—	25·0	—	—	25·1	—	24·5	24·7	24·6	—
5	—	—	25·0	—	—	24·9	—	24·6	24·6	24·5	24·0
10	24·9	—	25·1	—	—	24·6	—	—	24·7	24·5	23·5
15	25·1	—	25·4	—	—	—	—	24·1	24·9	—	—
20	25·1	—	25·4	—	—	—	—	—	25·1	—	23·4
30	25·4	—	—	—	—	24·4	—	24·1	25·0	24·0	—
					0·12 mg. P (theoretical reading 16·7 mm.)						
2	—	16·5	16·9	16·8	16·5	—	—	—	—	—	—
5	16·9	16·7	16·8	16·7	16·7	16·9	16·6	—	—	—	—
10	17·0	16·6	16·9	16·6	16·5	16·5	16·3	—	—	—	—
15	17·0	16·5	17·0	16·6	16·5	16·5	16·4	—	—	—	—
20	17·1	16·5	—	16·5	16·6	16·5	16·4	—	—	—	—
30	17·1	16·6	17·0	16·6	16·5	16·6	16·4	—	—	—	—
					0·25 mg. P (theoretical reading 8·0 mm.)						
2	—	8·1	—	—	8·1	—	—	—	—	—	—
5	8·3	8·1	8·1	8·2	8·1	8·4	8·5	—	—	—	—
10	8·2	8·0	8·1	8·1	7·9	8·2	8·2	—	—	—	—
15	8·3	8·1	8·1	8·1	7·9	8·1	8·1	—	—	—	—
20	8·2	8·0	8·1	8·1	7·9	8·1	8·1	—	—	—	—
30	8·2	8·0	8·1	8·1	8·0	8·0	8·1	—	—	—	—

Total phosphate. The sample is measured into a boiling-tube of about 1 × 6 in. and of good acid-resistant glass, such as monax or pyrex. 1·2 cc. of 60 % perchloric acid are added and the contents of the tube heated with a micro-burner or on an electric heater[1]. The contents of the tube become concentrated, turn brown and then, as the temperature rises and the acid begins to fume they become colourless, the organic matter being completely oxidised in a few minutes. In some cases where the amount of organic material is large and the oxidation slow it may be necessary to add a drop or two of nitric acid or of 30 % hydrogen peroxide; in this case it will be necessary to continue the heating for 3 or 4 minutes after the mixture has become colourless, in order to drive off the excess of nitric acid or hydrogen peroxide. The cooled contents of the tube are washed with several portions of water into a 15 cc. volumetric flask.

It has been found that about 0·2 cc. of the perchloric acid is lost during the course of heating in an average determination. While the loss of this amount of perchloric acid will not appreciably affect the colour produced, it is thought advisable to add this amount of extra acid in order to attain approximately the same concentration as that in the standard, and to insure against the possibility of overstepping the safe limits of variation in acidity.

1 cc. of molybdate and 0·5 cc. of sulphonic acid are added to the test and at the same time a standard is prepared from an appropriate number of cc. of the dilute standard solution, 1·2 cc. of perchloric acid, 1 cc. of molybdate and 0·5 cc. of sulphonic acid. Test and standard are diluted to the mark, mixed, and read after 5 minutes.

Some results with the method, using perchloric acid in the presence of several interfering substances, are given in Table I. The non-interference of

Table II. *Results of analyses of barium salts of phosphoric esters by perchloric acid method.*

Substance	P present (calc.) mg.	P found mg.	P theoretical %	P found %
Glycerophosphate	0·1010	0·1000 0·0996	10·1	10·0 9·96
Glycerophosphate, H_2O	—	—	9·55	9·45 9·62
Ethyl phosphate	—	—	11·88	11·87
Ethyl phosphate, H_2O	0·2903	0·2914 0·2941	11·11	11·13 11·24
Ethyl phosphate, H_2O	0·1443	0·1439 0·1419	11·11	11·08 10·93
Phenyl phosphate, $2H_2O$	0·0942	0·0938 0·0940	8·98	8·95 8·97
Phenyl phosphate, $2H_2O$	0·0931	0·0930 0·0932	8·98	8·97 8·99

[1] A very convenient electric heater has been described by Stanford and Wheatley [1925]. The elements for this heater can be obtained from Messrs Leonard Toomer and Co., Corporation Chambers, 54 Lower Thames Street, London, E.C. 3.

various substances with the colour produced with 1:2:4-aminonaphtholsulphonic acid when sulphuric acid is used has been amply demonstrated by Fiske and Subbarow and by Vasarhelyi [1929]. Table II contains the results of total phosphorus determinations on the barium salts of several phosphoric acid esters in which the organic material was destroyed by digesting with perchloric acid.

SUMMARY.

A procedure is proposed for the colorimetric determination of phosphorus in which sulphuric acid is replaced by perchloric acid, which is a much better oxidising agent for the destruction of the organic material in total phosphorus determinations. The colour is developed at a high acidity which allows of considerable variation without any loss in the proportionality of colour produced; hence no allowance is necessary for the presence of moderate amounts of trichloroacetic acid in test solutions. The full complement of blue colour is brought up in about 5 minutes by the use of 1:2:4-aminonaphtholsulphonic acid as the reducing agent.

REFERENCES.

Bell and Doisy (1920). *J. Biol. Chem.* **44**, 55.
Briggs (1922). *J. Biol. Chem.* **53**, 13.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Martland and Robison (1926). *Biochem. J.* **20**, 847.
Stanford and Wheatley (1925). *Biochem. J.* **19**, 697.
Vasarhelyi (1929). *Mikrochem. Pregl Festschrift*, 327.